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Antibacterial and Antibiofilm Effects of Green Copper Oxide Nanoparticles Synthesized using *Cotoneaster* Extract against *Enterococcus faecalis*

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A B S T R A C T

Objective: In nanotechnology, nanoparticles (NPs) are nowadays recognized as a beneficial tool for dental treatments. Metal NPs, especially copper oxide (CuO) NPs, have the potential for preventing and treating bacterial dental infections. This study was designed to assess the antimicrobial efficacy of CuO NPs synthesized using the aqueous extract of *Cotoneaster* plant against biofilm-producing *Enterococcus faecalis*.

Methods: For this purpose, we used the extract of the aforesaid plant and a green method to synthesize CuO NPs. The NPs synthesized herein were assessed using FTIR, XRD, UV-Vis, and SEM. Likewise, their antimicrobial and antibiofilm effects against *E. faecalis* were examined using the microdilution method.

Results: NPs were found to have sizes of 20-100 nm, in addition to having a monoclinic crystal structure. The synthesized NPs and chlorhexidine displayed minimum inhibitory concentrations of 1.25 mg/mL and 31.25 µg/mL, respectively. In antibiofilm testing, the NPs led to a relative reduction in biofilm formation and bacterial growth.

Conclusion: Overall, our findings suggest that green-synthesized CuO NPs derived from *Cotoneaster* extract could be an option for controlling microbial infections due to their strong antimicrobial and antibiofilm effects.

Keywords: *Enterococcus faecalis*, Copper oxide, Biofilm, Nanoparticles

➢ **How to cite this paper**

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Introduction

Dental caries is one of the most common oral diseases that occur due to the activity of oral microbes and the formation of biofilm [1]. Recent research advances in the field of dentistry have focused on manufacturing and applying various nanoparticles (NPs) for effective treatments. However, the development of biocompatible biomaterials with persistent efficacy in the mouth is still a main challenge in this research area [2,3]. Owing to ongoing difficulties in the domain of dentistry, growing interest has been devoted to the synthesis of NPs for various dental applications. These interests arise from the unique characteristics of NPs, including their very small size, biocompatibility, and favorable physicochemical properties [4,5]. In addition, NP biosynthesis has received the attention of researchers due to its advantages such as the use of natural reducing agents, stabilization of materials without toxic agents, low costs compared to expensive chemicals, and reduction of energy consumption.

The use of plants and living organisms such as bacteria, fungi, and algae to produce NPs is considered an eco-friendly method [6, 7]. Using plant extracts in the synthesis of NPs is often simpler and cheaper than using plant tissues, as these extracts play a reducing and controlling role in the growth of NPs. Different plant extracts are selected based on their chemical composition to influence the final properties of NPs [8]. *Cotoneaster* spp. is an herbaceous member of the Rosaceae family native to Europe and Asia, including Iran. These plants contain substances, such as tannins and flavonoids, which have medicinal properties and can be used in industries. This plant is especially abundant in temperate regions and foothills of Iran and typically thrives in environments with light and semi-humid soils [9, 10].

In medical science, copper is considered an anti-inflammatory and antimicrobial agent. Likewise, copper oxide (CuO) NPs have been reported to have antibacterial and antibiofilm characteristics [11,12]. These NPs exert their antibacterial effects through three main mechanisms: disruption of cell membranes, production of reactive oxygen species, and reduction of ATP production [13,14]. By penetrating the cells and interacting with molecules containing phosphorus and sulfur, such as DNA, CuO-NPs change the cellular structure and cause cell death. They also affect enzyme activity and produce hydrogen peroxide, which contributes to the leakage of DNA, RNA, and proteins from the cell membrane, ultimately leading to cell damage [14,15]. CuO-NPs, owing to their antimicrobial and physicochemical properties, can be used as a substitute for dental materials in clinical dental applications. These NPs are used in various dental materials such as dental metals and alloys, polymers, resins, and dental cements [16].

Previous studies have suggested a reduction in microbial biofilm due to the presence of NPs in dental materials [17]. The current study aimed to assess the antimicrobial and antibiofilm effects of green-synthesized CuO-NPs derived from *Cotoneaster* aqueous extract against biofilm-producing *Enterococcus faecalis*, a tooth root pathogen.

Materials and Methods

Preparation of *Cotoneaster* aqueous extract

The Cotoneaster leaves were obtained from a local herbal store (Attari) in Khorramabad, Iran. The leaves were washed with tap water and then distilled water. To obtain the Cotoneaster aqueous extract, we extracted dried plant material (10 g) with 100 mL of distilled water at 45°C for 90 min, then sonicated in a bath for 15 min. Afterward, the Cotoneaster extract was filtered through Whatman No. 1 filter paper. The filtrate was used to synthesize CuO NPs.

Biosynthesis of CuO NPs using *Cotoneaster* aqueous extract

CuO-NPs were fabricated using Cotoneaster extract as a capping and reducing agent, as described by Shah et al. (2022) under optimal conditions [18]. In a 250-ml flask, 50 mL of Cotoneaster extract was

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added to 50 mL of $CuSO_4 \cdot 5H_2O$ (0.1 M) solution. The pH of the reaction mixture was adjusted to 10 using sodium hydroxide (1 M), while stirring on a magnetic hot plate at 65 °C. After that, the sample solution was stirred continuously using a magnetic stirrer at room temperature for 24 hours. As CuO NPs formed, the solution turned dark brown. Furthermore, UV-Vis spectroscopy was performed to confirm CuO NP formation. The CuO NPs were centrifuged at 10,000 rpm at 4 °C for 15 min. To dry the CuO NP precipitate, they were washed twice in DW and then dried in an oven at 100 °C. The final CuO NPs were formed by calcining at 450 °C for 6 h. Finally, the produced CuO NPs were characterized by FTIR, XRD, and SEM imaging [17,18].

Antibacterial effect of biosynthesized CuO-NPs against *E. faecalis*

Antibacterial activities of CuO NPs at concentrations (50, 25, 12.5, and 6.25 mg/mL) were examined based on the agar well diffusion method. Furthermore, chlorhexidine at 4 diluted concentrations (20, 10, 5, and 2.5 µg/mL) was used as the comparative positive control. To prepare the experiment, sterile MHA plates were created, and E. faecalis ATCC 29212 was evenly spread on the agar surfaces at a cell density of 0.5 McFarland standard (1.5×10^8 cfu/mL) using sterile cotton swabs. Afterward, we created 4 wells (about 40 µL) with a 6-mm steel punch for each concentration on the 8-cm agar plates. The wells were filled with 40 µL of each concentration of Cu NPs or chlorhexidine and incubated in a bacterial incubator at 37 °C for 24 h. Finally, growth inhibition zones were measured using a digital ruler as zone inhibition diameters [19]. All treatments and dilutions were performed in at least three replicates to ensure reliable statistical analysis of the mean results.

MIC and minimum bactericidal concentration (MBC) of NPs

To determine the MIC of synthesized CuO nanoparticles and CHX, a microdilution method was used in 96-well plates [20]. The wells were inoculated with different concentrations of nanoparticles or CHX, 10 µL of *E. faecalis* at half McFarland concentration, Mueller-Hinton broth culture medium, then the plates were incubated for 24 h at 35–37 °C. The MIC synthesized CuO nanoparticles and CHX was determined by observing the color change in the wells using 10 µL of TTC reagent (20 mg/mL). To determine the MBC, we used the MIC and higher concentrations. To determine the minimum bactericidal concentration (MBC), wells within the MIC range were cultured on MHA agar plates and after 24 hours of incubation, the lowest concentration that inhibited bacterial growth was reported as the MBC [21]. The experiment was performed in triplicate.

Qualitative study of the antibiofilm activity of synthesized NPs

To evaluate the antibiofilm activity, we placed a sterile glass slide in a sterile Falcon tube containing 10 mL of Mueller-Hinton Broth culture medium supplemented with 3% glucose, along with varying concentrations of the synthesized NPs and 50 µL of microbial suspension equivalent to half the McFarland standard of *E. faecalis*. The same test was performed for CHX. The incubation of tubes was done at room temperature for 16 h, and the slides were washed with 0.9% normal saline. All the stained slides (0.1% crystal violet) were screened with a microscope [22].

Results

In biosynthesizing CuO NPs using the *Cotoneaster* aqueous extract, the color change of the reaction mixture was observed as an indicator to confirm NP formation. This color change usually shifts from blue green to dark brown and signifies the synthesis of CuO NPs successfully.

UV-Vis spectrophotometric results of synthesized CuO NPs

The UV-Vis spectrophotometric analysis revealed that NPs often exhibit a characteristic absorption peak in the wavelength range of 500 to 600 nm. The absorption spectra of the *Cotoneaster* aqueous extract and the synthesized NPs are shown in

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Figure 1 (red and black colors, respectively). The red spectrum did not show any specific absorption peak, but the black spectrum did show one at a wavelength of 534 nm. The UV–Vis findings were interpreted in conjunction with the FTIR and SEM/EDX characterization results, which collectively support the formation of the synthesized CuO NPs.

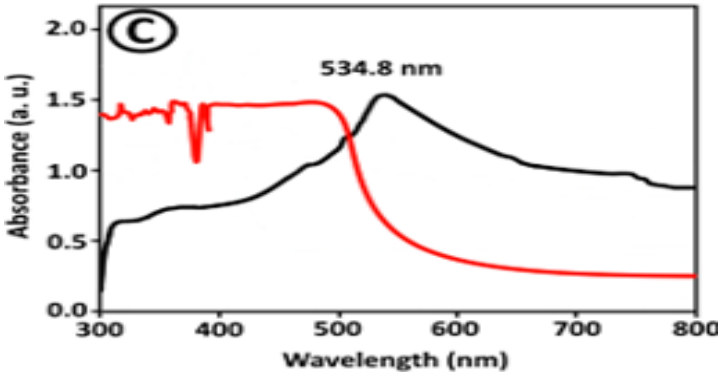
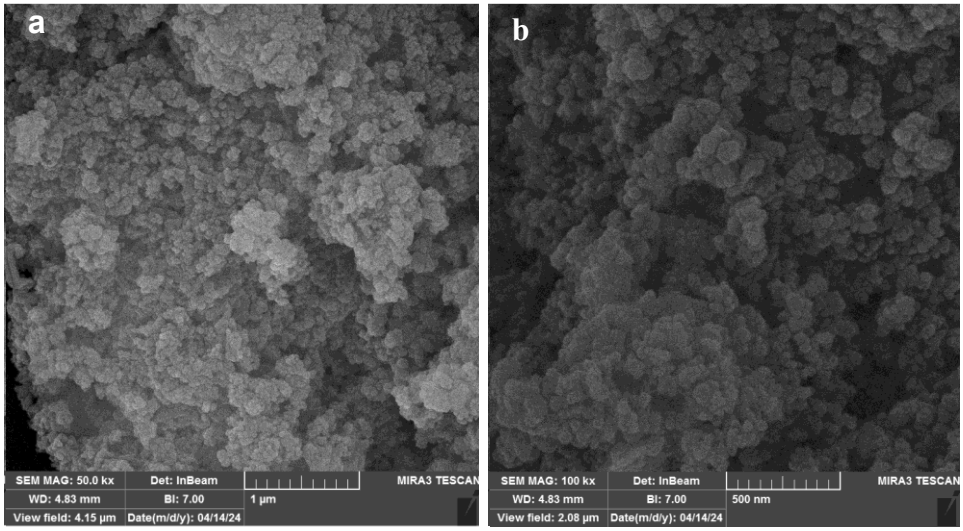


Figure1: UV-visible spectrophotometric analysis of the production of CuO NPs using *Cotoneaster* aqueous extract

SEM imaging

The analysis of images using SEM showed that the synthesized NPs were mostly spherical in shape and individually clustered. The dimension of such NPs was found to be in the range of 20-100 nm, and the average particle size was 60 nm. Furthermore, the presence of copper and oxygen was confirmed by the EDX, and the weight percentage was determined (Fig. 2).



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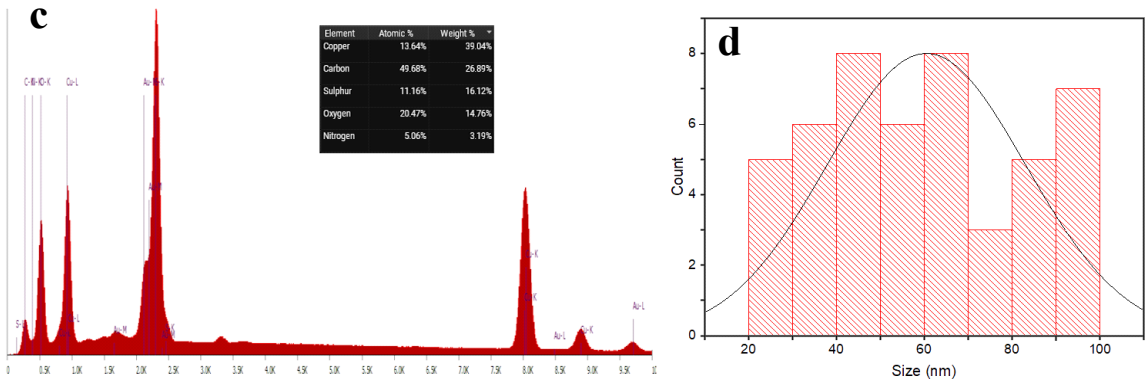

Figure 2: SEM image (a, b), size distribution analysis (c), and EDX analysis (d) of synthesized CuO NPs.

XRD results

XRD patterns were obtained using an XRD Normal Rigaku Ultima IV with CuKα radiation (λ = 1.54059 Å), scanning 2θ angles from 10 to 80°. As seen in Fig. 3, the XRD spectra provide major intense peaks at 2θ values of 32.5°, 35.5°, 38.7°, 48.7°, 53.5°, 58.3°, 61.5°, 66.2° and 68.0° with miller planes indexing to (110), (002), (111), (202), (020), (202), (113), (311) and (220), which correspond to monoclinic crystalline structure and matching the JCPDS card no. 05-0661 [23]. According to the study, the characteristic peaks at 32.6° (plane -110), 35.8° (plane 002), and 38.9° (plane 111) align with the standard pattern. However, additional peaks may appear due to the synthesis method and metabolite interference. In this study, while plant extract compounds acted as stabilizing/capping agents, the CuO NPs were approximately 88% crystallinity degree. Furthermore, the average crystallite size, calculated from the characteristic peaks using the Scherrer method, was approximately 15.35 nm. In this regard, the discrepancy between the crystallite size determined by XRD (15.35 nm) and the particle size observed via SEM (approximately 60 nm) can be attributed to the fundamental difference between these two parameters. Based on the results, the XRD pattern reflects the crystalline units of the nanoparticles, whereas the size estimated from SEM images includes contributions from coating and stabilizing agents.

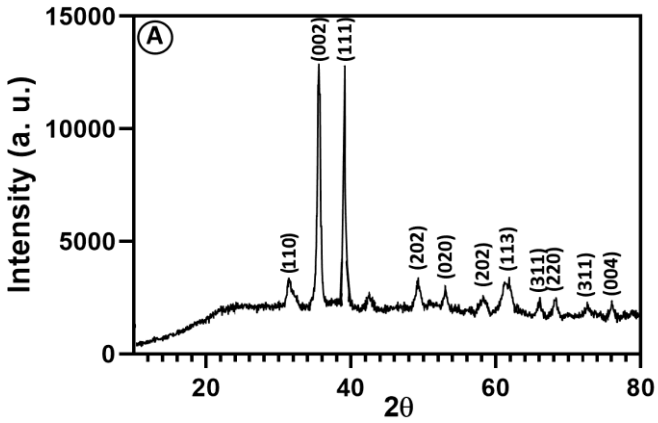

Figure 3: XRD pattern of CuO NPs synthesized by the *Cotoneaster* aqueous extract

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FTIR analysis

The FTIR spectrum of the aqueous extract of the plant and the green-synthesized CuO NPs is shown in blue and black, respectively (Fig. 4). The spectrum of the Cotoneaster extract shows the presence of hydroxyl (OH), amine (NH), and carbonyl (C=O) functional groups. The spectrum of CuO NPs exhibits significant shifts compared with the spectrum of the plant extract, particularly in some functional groups, suggesting their possible involvement in the interaction with and stabilization of the synthesized nanoparticles. One important band in the FTIR spectrum of CuO NPs is observed at 491.2 cm^{-1}, which can be attributed to the Cu–O vibration, confirming the formation of CuO. The changes in the positions and intensities of the bands corresponding to OH, NH, and C=O groups may indicate their involvement in the reduction and stabilization of CuO NPs during the green synthesis process. However, FTIR analysis alone does not provide sufficient evidence to assess the crystallinity or quality of the synthesized nanoparticles.

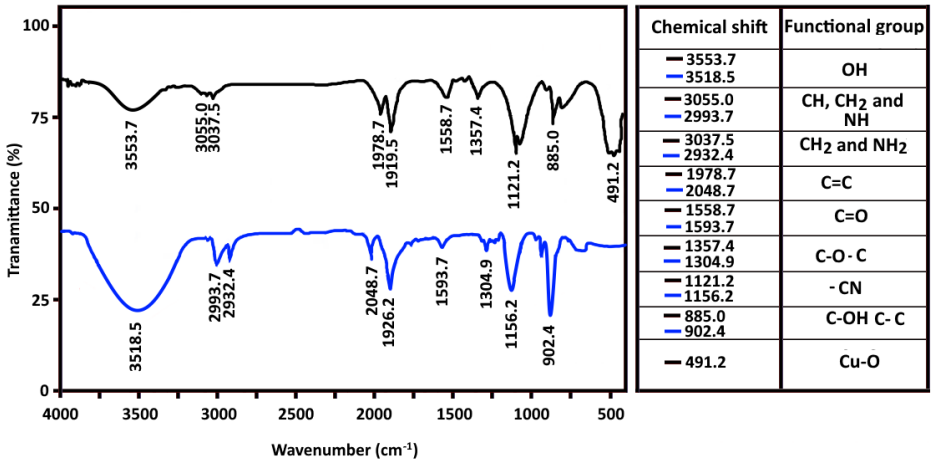

Figure 4: FTIR spectroscopic pattern of CuO NPs synthesized by *Cotoneaster* aqueous extract

Results of antimicrobial properties of CuO NPs

The non-growth zone formed through the treatment of *E. faecalis* in different concentrations of CuO NPs (50 to 6.25 mg/mL) and CHX (20 to 2.5 µg/mL) showed that the growth inhibition pattern was concentration-dependent (Fig. 5). Also, the highest concentrations of NPs (50 mg/ml) and CHX (20 µg/mL) were the most effective in inhibiting the growth of bacteria. We found that the no-growth zone diameter was 20 mm for CHX at 10 µg/mL and NPs at 50 mg/mL.

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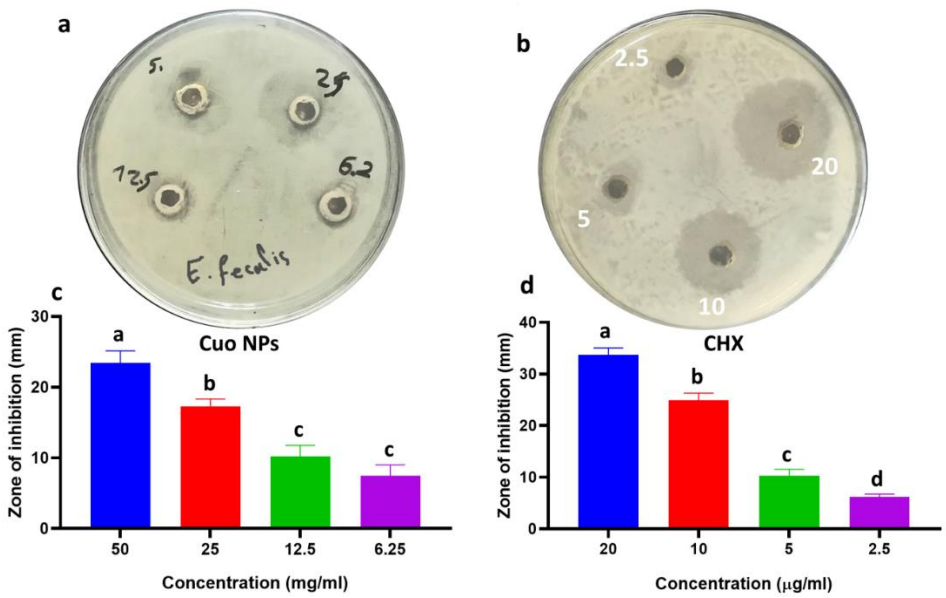

Figure 5: Antibacterial activity assay of a) Cuo NP and b) chlorhexidine at four concentrations, zone of inhibition of c) CuO NPs and d) Zone of inhibition of chlorhexidine against *E. faecalis*. Data are presented as mean ± SD from three replicates. Different superscripts display differences between treatments (p-value < 0.05)

Results of the MIC and MBC of synthesized CuO NPs compared to CHX

Based on the results of MIC and MBC, as depicted in Table 1and Fig 6, the aqueous extract of the plant showed weaker antimicrobial activity for CHX than that for the synthesized CuO NPs.

Table 1: MIC and MBC of CuO NPs, plant extract, and CHX

Compounds	MIC	MBC
Aqueous plant extract	-	-*
CuO NPs	1.25 mg/mL	1.25 mg/mL
CHX	31.25 µg/mL	31.25 µg/mL

*-: Not observed

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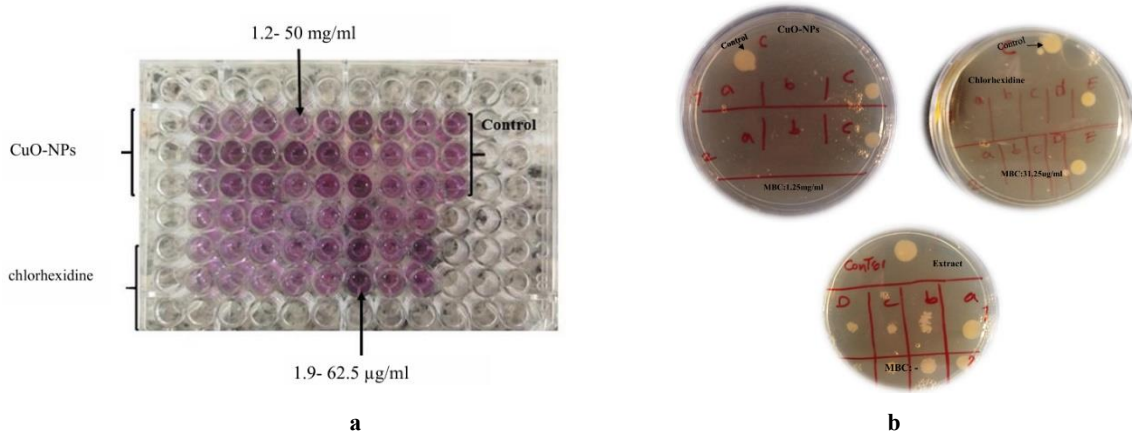

Figure 6: MIC (a) and MBC (b) of synthesized CuO NPs compared to CHX against *E. faecalis*

Antibiofilm activity of CuO NPs against *E. faecalis*

Figure 7 depicts a comparison of the antibiofilm activity of CuO NPs, CHX, and untreated control. Biofilm formation on the slides, shown in Figures 7a, 7b, and 7c, was quantified by density using ImageJ and converted to percentages. These data revealed a 76% reduction in biofilm in the chlorhexidine-treated group. Meanwhile, the nanoparticle-treated group showed an approximate 87% reduction in biofilm formation. Although the values were calculated semi-quantitatively, statistical analysis indicated a significant difference between the CuO NPs-treated group and the chlorhexidine group. This observation showed that CHX and CuPO NPs were very effective at inhibiting biofilm formation. The untreated control exhibited a significant abundance of bacteria, regardless of the level of concentration, with the bacteria being well-dispersed (Fig. 7c).

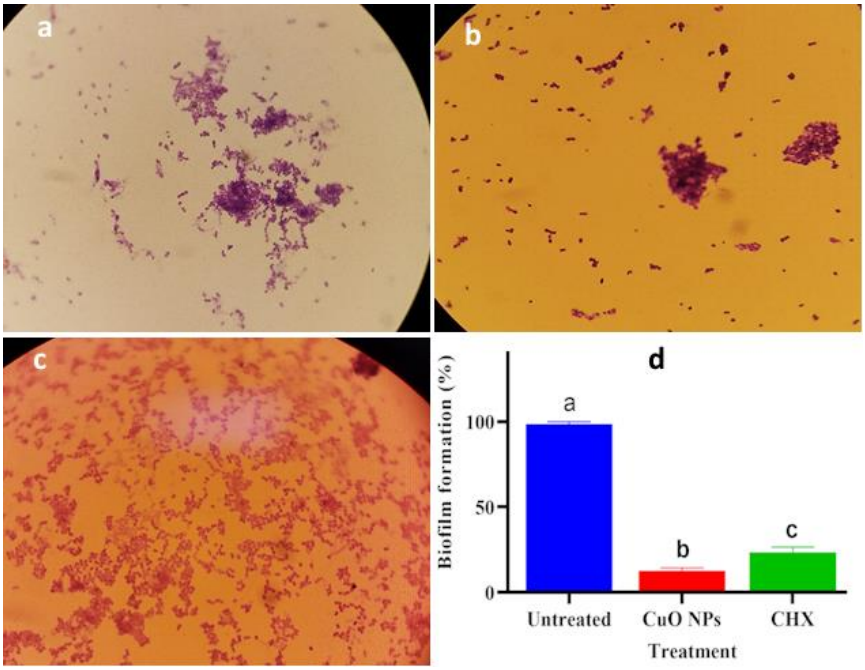

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Figure 7: Antibiofilm effect of (a) CuO NPs, (b) CHX, (C) untreated control and (d) biofilm density as percentage calculated based on qualitative biofilms using image J software. Different superscripts display differences between treatments (p-value < 0.05)

Discussion

In the present study, we designed and utilized CuO NPs to study the antimicrobial and antibiofilm impact of these NPs prepared using a green approach using *Cotoneaster* extract. The color change of the solution to dark brown after the sonication process proved successful in the synthesis of CuO NPs, similar to the color change observed in Ahmad et al.'s green synthesis process using *Aloe vera* extract [24]. Further, the UV-Vis absorption data exhibited a single peak at 534 nm, the SEM image revealed the shape and size of the synthesized CuO NPs, and the FTIR data indicated the presence of functional groups, signifying the successful synthesis of green synthesis of CuO NPs.

In this study, by examining the bacterial growth inhibition zone, we found that both CuO NPs and plant extract could inhibit the growth of *E. faecalis*, but at higher concentrations compared to CHX. The results of MIC and MBC also showed the antimicrobial effectiveness of these compounds, again at levels higher than those required for CHX. The results of antibiofilm images also indicate the same results. In a study by Awad et al. in 2019, the green synthesis of CuO NPs with *Ailanthus altissima* plant extract also demonstrated antibacterial properties. Their study found a light absorption peak at 396 nm, probably because they used different plant material [25]. The antibacterial activities of the *Eupatorium adenophorum* extract-mediated CuO NPs were studied against *Staphylococcus aureus*, *Escherichia coli*, *E. faecalis*, *and Salmonella typhi*. The results obtained in their study were matched with those of our research [26].

Numerous earlier studies have shown that NPs have both antibacterial and antibiofilm properties. In a study by Oetiker et al. in 2024, the antibiofilm effect of Cu^0, Cu_2O, and CuO-NPs was reported against *Streptococcus mutans* [27]. Agarwala et al. reported the antibiofilm activity of CuO NPs against *E. coli* isolates [28]. In another recent investigation, Bai et al. further reported an antibacterial property of green-synthesized CuO NPs against *Acinetobacter spp.*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* [29]. The antimicrobial activity of CHX is very high at low concentration, and considerable research is currently in progress to identify other antimicrobial agents that are effective, such as green-synthesized NPs. NPs—especially the green-synthesized CuO NPs—have special advantages that validate the use of such compounds. They exhibit a broad spectrum of effects against bacteria and fungi, while CHX is effective only against bacteria. Other notable characteristics of these NPs include their stability and prolonged persistence; however, the potential for development of microbial resistance to CuO NPs remains to be established and requires further experimental investigation. Moreover, CuO-NPs are modifiable, can be combined or loaded to increase their efficacy and/or decrease toxicity, and can work through several mechanisms such as generation of reactive oxygen species, ion release, or destruction of membranes.

The green synthesis of CuO NPs from an economic point of view is primarily less expensive and more eco-friendly, which could benefit applications where natural materials are required or where the use of strong chemical compounds is not allowed.

Thus, the CuO NPs synthesized in this study are considered a candidate for environmental application. As a result of the present research, further studies to assess the antibacterial activity of these NPs in vivo and additional testing could yield these NPs as effective antibacterial and antibiofilm agents.

Conclusion

An aqueous extract of the *Cotoneaster* plant successfully green synthesizes CuO NPs that have antibacterial and antibiofilm activity against *E. faecalis* in vitro. Unlike this metal compound, CHX

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had the highest inhibitory effect at very low concentrations. Likewise, the plant extract indicated the weakest performance, with only antimicrobial and antibiofilm effects.

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Ethics

The study, conducted under the ethical code: IR.LUMS.REC.1403.146, was approved by the Ethics Committee of Lorestan University of Medical Sciences, Lorestan, Iran.

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None.

Competing interests

The authors have no conflicts of interest.

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